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# Evolution of Bacteriophage T7 in a Growing Plaque

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**The emergence of mutants during the $10^9$-fold amplification of a bacteriophage was spatially resolved in a growing plaque. When wild-type phage T7 was grown on an *Escherichia coli* host which expressed an essential early enzyme of the phage infection cycle, the T7 RNA polymerase, mutant phage relying on this enzyme appeared in $10^8$ phage replications and outgrew the wild type. Spatial resolution of the selection process was achieved by analyzing stab samples taken along a plaque radius. Different mutants were selected at different rates along different radii of the plaque, based on host range and restriction patterns of the isolates. The mutants deleted up to 11% of their genomes, including the gene for their own RNA polymerase. They gained an advantage over the wild type by replicating more efficiently, as determined by one-step growth cultures.**

More than 10 years ago, Studier (25) proposed bacteriophage T7 as a model system to study phage evolution and phage ecology. As attractive characteristics, he cited its rapid growth rate, the easy discrimination of closely related phage strains by restriction analysis, the conservation of a T7-like genetic and functional organization among a broad spectrum of phage isolates (13), and the straightforward generation of phage mutants by growth in serial transfer cultures. Recently, the system was used to experimentally generate a predetermined phylogeny, on which methods of phylogenetic inference could be compared (15). This study demonstrates that the phage's ability to grow continuously in a plaque provides additional motivations for adopting T7 to study phage evolution.

When phages T1 through T7 were first characterized nearly 50 years ago, T7 was already recognized as a large-plaque former (6). Its plaque size, however, was only defined relative to the size of other T-phage plaques after a prescribed incubation period. Recently, it was observed that T7 plaques can grow indefinitely large (30); the borders of the petri dish, collision with other plaques, and dehydration of the plating agar pose the only limitations to unbounded growth. Typically, a growing plaque may be observed for 120 h, during which a single phage reproduces $10^{10}$-fold while generating a plaque greater than 5 cm in diameter.

The plaque system offers several distinctive features for observing the growth and evolution of phages. First, differences among phage isolated from a plaque reflect an emergent diversity. Heterogeneity of the inoculum is not an issue since each plaque is initiated by a single virion. Second, the plaque is an accessible historical record. As they multiply, phage populations are fixed in ever-expanding concentric rings, each ring corresponding to the plaque circumference at a point in time; sampling across rings can reveal events in time. Third, the rate of plaque growth is a quantifiable phenotype for the continuously replicating phage. It depends on environmental factors such as the state of the host, temperature, and the presence of agents that interact with the phage or its genome. A theoretical model has been developed which shows how this rate depends on the microscopic phenomena of phage adsorption, replication, release, and diffusion (31); a conceptually similar system was originally developed to study the in vitro replication and evolution of RNA molecules (3). Finally, from a practical perspective, the system is inexpensive and easily implemented.

As an initial test of the system, the wild-type growth cycle was perturbed by plating wild-type T7 on a host that expressed the T7 RNA polymerase. This enzyme plays an essential role in T7 transcription (5) and is synthesized within the first 6 min of an infection cycle that lasts 25 min at 30°C (26). When the enzyme is expressed in a recombinant host, patterns of phage protein synthesis are shifted forward several minutes following a wild-type infection (18), suggesting that the host-expressed enzyme is immediately used by the entering wild-type DNA. Further, T7 mutants lacking the gene for their own RNA polymerase or carrying amber mutations in genes involved with virion structure and assembly can grow on complementing hosts (4, 18, 24, 28), demonstrating that phage genes essential for growth need not be carried by the phage. By plating wild-type T7 on the recombinant host, I grant the phage an opportunity to skip an indispensable step of its natural growth schedule. One may then observe how populations in the growing plaque adapt in this unnatural environment.

## MATERIALS AND METHODS

Established methods were used in the preparation, preservation, and concentration determinations of the phage and bacteria (1, 17, 23). The wild-type strain of phage T7 as well as the host strains *Escherichia coli* BL21 (supports growth of T7 defective in genes 0.3 and 0.7), BL21(DE2) (supports growth of T7 defective in gene 1), and BL21(DE3) (supports growth of T7 defective in gene 1 [under IPTG control]) were provided by F. W. Studier (27). To observe phage evolution, I prepared plates in a single agar-nutrient host layer as previously described (30).

**Phenotypic selection profiles.** Wild-type T7 was plated on BL21(DE3) in the presence of 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG; Stratagene, La Jolla, Calif.). After 50 h of incubation at 37°C, 12 phage populations were isolated from a single plaque by collecting stabs at equal spacings along the plaque periphery; a fresh sterile pipette

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tip was used for each stab and then washed in 1 ml of phage buffer (10 mM Tris-HCl [pH 7.5], 1 mM $MgCl_2$, 0.1 M NaCl, 10 g of gelatine per liter); these samples typically contained $10^5$ to $10^6$ PFU.

The stab solutions were tested for the presence of the host-dependent phenotypes by double-layer plating on host strains BL21(DE2) and BL21. An aliquot of the solution was mixed with BL21(DE2) in soft agar and plated on a layer of nutrient agar and allowed to harden; then a layer containing only BL21 in soft agar was poured over the previous layer and incubated for 4 to 6 h at 37°C. Mutants which rely on the host-expressed T7 RNA polymerase grow on BL21(DE2), but not on BL21; hence, they could be distinguished from the wild-type phage by their turbid-plaque rather than clear-plaque phenotype. This assay is predisposed to detect deletion mutations rather than point mutations, which would be revertible. The fraction of host-dependent mutants in a stab sample was given by the number of turbid plaques divided by the total number of plaques, turbid and clear. Error bars were determined by assuming an error of $M^{1/2}$ for $M$ plaques counted and propagating these errors in calculations (2).

The histories of 3 of the original 12 phage populations sampled from the plaque perimeter were studied in detail. Additional stab samples were taken along radii 1, 5, and 9 at 1-mm intervals from the center to the edge of the plaque (see Fig. 1E). By double-layer plating, the fraction of the population requiring the host-expressed T7 RNA polymerase was determined for each isolate.

**Genotypic selection profiles.** Phage populations from stab solutions obtained along different plaque radii were amplified on the host used for plaque growth, BL21(DE3) with IPTG induction of the T7 RNA polymerase. Typically, 0.1 ml of stab solution was used to inoculate a 25-ml shaker culture having a density of $10^8$ cells per ml in 10 g of nutrient broth (DAB 7; Merck) per liter containing 0.4 mM IPTG. Phages were concentrated with polyethylene glycol 6000 and heat denatured at 70°C in 4% sodium dodecyl sulfate; proteins were removed by centrifugation after precipitation in potassium acetate. The resulting solubilized DNA was adsorbed onto an anion-exchange resin (Tip-100; Qiagen), eluted, precipitated with isopropanol, and redissolved in 0.5 ml of universal buffer (0.1 M potassium acetate, 25 mM Tris-acetate [pH 7.6], 10 mM magnesium acetate, 10 mM β-mercaptoethanol, 10 μg of bovine serum albumin per ml; provided with restriction enzymes from Stratagene). Detailed protocols are provided with the Qiagen ion-exchange cartridges.

Restriction patterns were produced by cutting the phage DNA with *Hpa*I (Stratagene) and separating by agarose gel electrophoresis (16). Gels (1.5% agarose [type II: medium EEO; Sigma]) in TBE buffer (18 mM Tris-HCl, 18 mM boric acid, 0.4 mM EDTA, pH 8.0) were typically run for 4 to 6 h at 5 V/cm, stained with ethidium bromide, transilluminated at 254 nm, and photographed through an appropriate filter (Wratten gelatin filter no. 16; Kodak).

**Isolation, amplification, and characterization of host-dependent mutants.** Clones of individual mutants were identified by their ability to form turbid plaques on double-layer plates, as described above. Although still newer mutants may conceivably emerge and be selected during the growth of such turbid plaques, this possibility was minimized by taking stabs from the center of plaques as soon as they could be unequivocally identified as turbid (3 to 5 h of incubation). These samples were further amplified in shaker culture, and their DNA was extracted as described above. Mutant clones

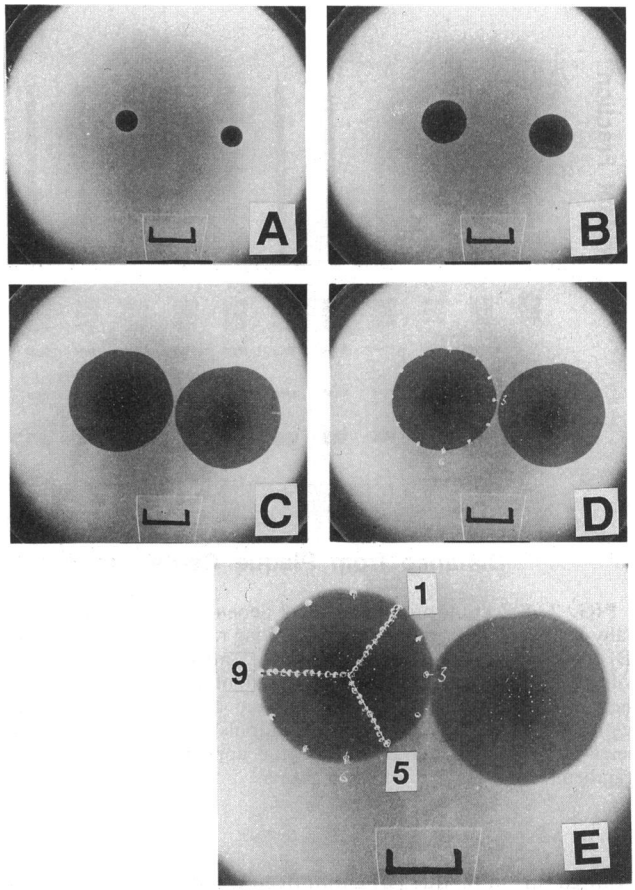


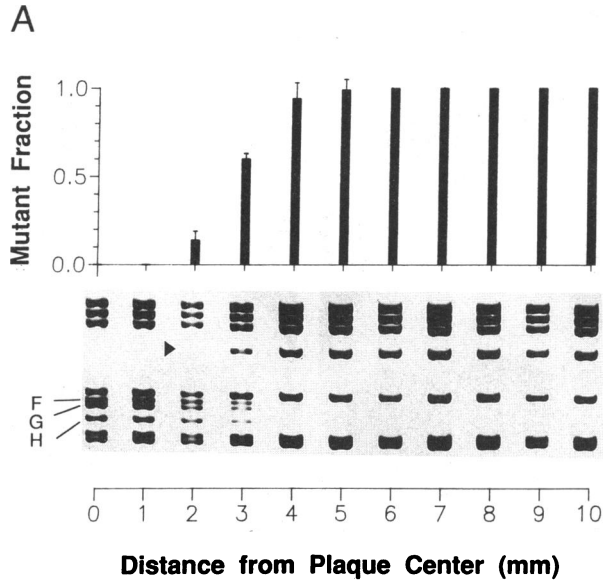

FIG. 1. Growth and sampling of a bacteriophage T7 plaque. Wild-type T7 was plated on BL21(DE3), a host that expresses the T7 RNA polymerase. Plaques are shown after 12 (A), 24 h (B) and 50 h (C) of incubation. The positions of 12 samples taken along the plaque periphery (D) and of adjacent samples along three radii (E) are shown. The rule is 1 cm long.

isolated along radii 1 and 5 were named p001 and p005, respectively.

Deletions within the genomic DNA of mutants were characterized by agarose gel electrophoresis of restriction digests with *Hpa*I, *Scr*FI, *Hae*II, and the double digest *Hpa*I-*Scr*FI (all enzymes for Stratagene). The patterns for *Hpa*I and *Hae*II are established (21), and the pattern for *Scr*FI was obtained by using the known sequence for T7 (8) and a VAX program for searching and comparing sequences (7).

The program was also used to propose sites of genetic deletion based on the observation that deletions in phage T7 frequently occur between directly repeated DNA sequences (20, 21). The approximate size and location of a deletion was determined by comparing the bands in mutant and wild-type restriction patterns. The size of a new band was estimated by interpolation between adjacent bands assuming a linear dependence between the band migration distance and the log of the number of base pairs (14). Subject to the established size and position, possibilities were tested by using the COMPARE routine (7); regions of highest homology were chosen as the most probable.

**Test for local genetic diversity.** Stab samples were obtained



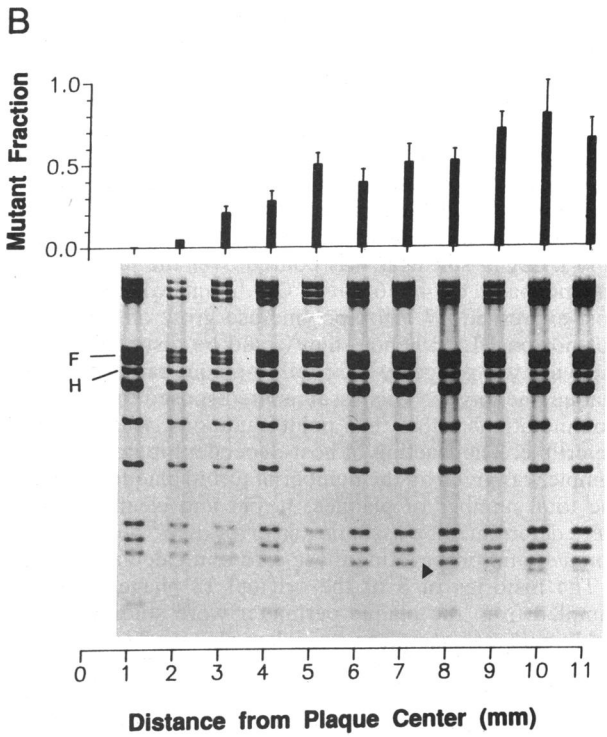
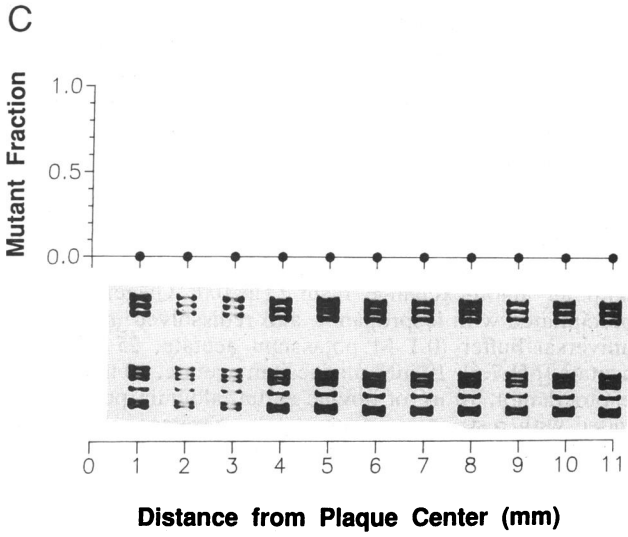




FIG. 2. Selection profiles for host-dependent T7 mutants. Spatially resolved emergence of mutants along radius 1 (A) and radius 5 (B) of the plaque in Fig. 1E is shown. In contrast, persistence of the wild type is found along radius 9 (C). Identification of mutants by their need for host complementation and the generation of *Hpa*I restriction patterns is detailed in Materials and Methods. Arrowheads in panels A and B indicate the appearance of new DNA fragments characteristic of the mutants.




from 10 turbid plaques at 2 mm from the plaque center along radii 1 and 5. These were amplified, and an *Hpa*I restriction pattern was obtained for each mutant as described above.

**One-step-growth cultures.** The method is adapted from that of Ellis and Delbrück (12). A 25-ml shaker culture of BL21(DE3) on 10 g of nutrient broth per liter with 0.4 mM IPTG was grown to an optical density of 0.6 at 600 nm, corresponding to $5 \times 10^8$ cells per ml. An aliquot of phage was added at a multiplicity of infection from 0.3 to 0.8, and the shaking was continued for 5 min. Then the sample was diluted 1,000-fold into a prewarmed flask containing 10 g of nutrient broth per liter, and the shaking was continued. Samples were then taken at 2-minute intervals, stored at 0°C until the sampling was completed, diluted, and plated out on BL21(DE2). Phage concentrations were determined by counting plaques after 4 to 6 h of incubation.

**Plaque propagation rates.** Phage samples were diluted and distributed on single-layer plates containing host BL21 (DE3), 10 g of nutrient broth per liter, 10 g of agar-agar per liter, and 0.4 mM IPTG. The concentration of host was $3 \times 10^6$ colonies per ml, fivefold lower than that used for the generation of selection profiles; lower host concentrations were used here to minimize the chances that new mutants could emerge (29). Plaque diameters were measured with a hand-held digital caliper (Helios-digit; Helios, Germany), always measuring at the same orientation, at 13, 18, and 23 h; plaques were chosen based on their large separation from other plaques and from the edge of the plating dish. For p001, p005, and the wild type, a total of 21, 27, and 11 plaques, respectively, were measured. The average radial propagation rate (millimeters per hour) was obtained as one-half of the slope from a linear regression for the three points.

Theoretical values for the radial propagation rates of p001 and p005 were determined by using a recently developed model (31). Assuming that slow-adsorption processes dominate the propagation behavior and that the mutant and wild-type virion diffusivities and rates of adsorption are identical, an analytical expression is obtained: $c_{mu}/c_{wt} = [(Y_{mu} - 1)/(Y_{wt} - 1)]^{1/2}$, where $c_{mu}$ and $c_{wt}$ are the radial propagation rates (or velocities) for the mutant and wild type, respectively, and $Y_{mu}$ and $Y_{wt}$ are the average yields of mutant and wild-type phage per infected host, respectively. By obtaining average yields of $Y_{wt}$, $Y_{p001}$, and $Y_{p005}$ from one-step growth cultures and $c_{wt}$ from the average of the measured wild-type propagation rates, values for $c_{p001}$ and $c_{p005}$ based on the model were calculated.

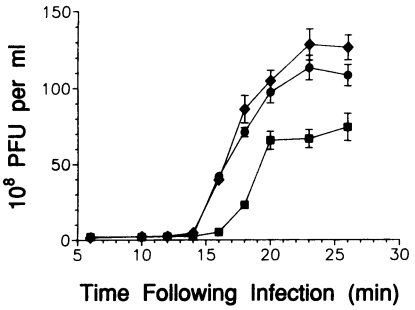



FIG. 3. One-step growth curves of T7 mutants. Plaque-derived mutants p001 (●) and p005 (◆) and the wild type (■) were grown in shaker cultures of BL21(DE3) in the presence of IPTG. After infection, the cultures were periodically sampled, and the samples were diluted, plated, and counted to obtain the growth curves.

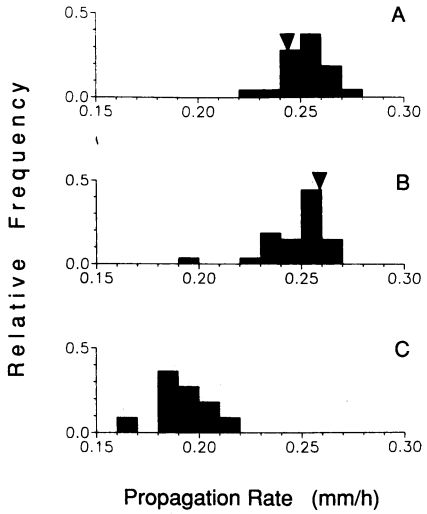



FIG. 4. Plaque propagation rates for T7 mutants. Rates of radial propagation (millimeters per hour) were determined for plaques of p001 (A), p005 (B), and wild type (C). Theoretical values for the average rates are shown for the mutants (arrowheads in panels A and B) based on the shaker culture results of Fig. 3.

## RESULTS

**Selection profiles.** The continuous growth of a plaque of wild-type phage T7 on BL21(DE3), a host which expressed the phage RNA polymerase, was monitored over 2 days, as shown in Fig. 1A to C). After 50 h of incubation, 5 of the 12 stab samples taken along the periphery of the plaque (Fig. 1D) contained phage mutants which required the complementing host for growth. Of the 12 samples, 3 at positions 1, 5, and 9 were selected for further characterization; they were composed of 100%, less than 100% and 0% mutant phenotypes, respectively.

Phage populations which preceded those at the plaque periphery were isolated by sampling additional points along radii 1, 5, and 9 (Fig. 1E) and testing for mutants. Along radius 1, the wild-type virion which initiated the plaque succeeded in maintaining its phenotype during the initial stages of plaque growth, as shown in Fig. 2A (upper panel); near the plaque center no mutants were detected. However, 2 mm from the plaque center, mutants were detected, and at increasing radial distances the mutant fraction increased until it completely outgrew the wild type. This displacement of the wild type was also visualized at the level of genotype by noting changes in the *Hpa*I restriction patterns for the sampled populations, as shown in Fig. 2A (lower panel). As the mutant fraction increased, the F, G, and H bands of the wild-type pattern faded out and a new band having a higher molecular weight appeared, corresponding to the emergence of a genetically distinct mutant, designated p001.

The wild-type population also encountered competition along radius 5, but this mutant, designated p005, emerged more gradually than p001, as shown in Fig. 2B (upper panel). At the genotype level, the gradual decrease in the wild-type fraction was reflected by the reduced intensity of the F and H bands and appearance of a new band having a lower molecular weight, as shown in Fig. 2B (lower panel).

In contrast to the pattern changes along radii 1 and 5, the wild-type population remained unchanged for all isolates along radius 9. No mutants were detected anywhere along radius, 9, as shown in Fig. 2C.

**One-step growth cultures and plaque propagation rates.** Figure 3 shows that mutants p001 and p005, which were isolated from the plaque shown in Fig. 1E, grew more rapidly in one-step growth cultures on induced BL21(DE3) than the wild-type phage which initiated the plaque. The latent periods in both cases were 3 min shorter and the average burst sizes were 60 to 80% higher than those of the wild type.

In plaques, mutants p001 and p005 also grew faster than the wild type, as shown in Fig. 4. Given the average burst yields for the mutants and the wild type from the one-step growth cultures and the average propagation rate measured for the wild type, theoretical values of average propagation rates for the mutants were determined (indicated by arrowheads in Fig. 4A and B) and found to fall within the experimentally measured rate distributions.

**Genotypic diversity of mutants.** To test whether the selected mutant along a given radius could be emerging from a pool of diverse mutants initially present at low concentrations relative to the wild type, individual mutants isolated from single stab samples containing low mutant fractions were amplified and tested. Based on their dependence on host-provided polymerase for growth, 10 mutants were isolated from the stab sample taken 2 mm from the plaque center along radius 1. They all produced the same *Hpa*I restriction pattern as the eventual winning mutant, p001 (Fig. 5, lane 2). Likewise, 10 mutants isolated at 2 mm from the plaque center along radius 5 also produced all the same *Hpa*I restriction pattern as the eventual winner, p005 (Fig. 5, lane 3).

In contrast to the homogeneity of mutant species within the local population of a stab sample, mutants isolated from different plaques were nearly all different. The diversity is reflected by the *Hpa*I restriction patterns for 10 of the 14 mutants isolated from six plaques, shown in Fig. 5. Only one mutant, p005 or 5A2a, was isolated twice. The deletions ranged from 1,200 to 4,400 bp, up to 11% of the T7 genome. On average, they deleted 83% of gene 0.7 and 62% of gene 1. All mutants except 3/10t deleted at least 22% of gene 0.7, and all mutants except 3/10c deleted at least 25% of gene 1. These two exceptions were isolated from the same stab. The wild type was not present in the stab population, based on the identical restriction patterns obtained for the entire stab amplified in the absence of gene 1 induction and from 3/10c (Fig. 5, lane 4).

Deletion mutants readily emerge during plaque growth on complementing hosts such as BL21(DE3) in the presence of

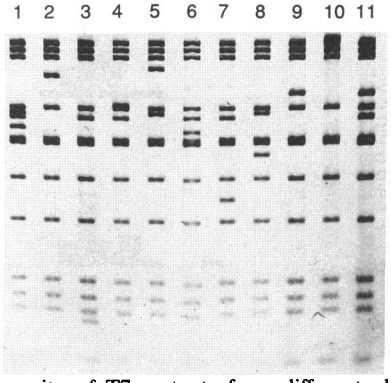



FIG. 5. Diversity of T7 mutants from different plaques. *Hpa*I restriction patterns are shown for wild type (lane 1) and mutants p001 (lane 2), p005 (lane 3), 3/10c (lane 4), 3/10t (lane 5), 3/19 (lane 6), 9B2a (lane 7), 10A3b (lane 8), 2A3 (lane 9), 2B3 (lane 10), and 7A2 (lane 11).

IPTG or BL21(DE2). From the study described above, 13 of 15 plaques of wild-type T7 plated on BL21(DE3) contained deletion mutants; an average of only five stabs were tested from each plaque. To date, 29 of 35 plaques of wild-type T7 plated on polymerase-complementing hosts have contained host-dependent mutants; 37 of 99 stab samples from these plaques contained mutants. Of the six plaques in which no mutants were detected, fewer than five stabs were tested from each; mutants may have gone undetected because of sparse sampling.

**Frequency of deletion.** The dimensions of the plaque at the time the mutants first appeared are used to estimate the frequency of deletion. Both mutants p001 and p005 were detected at a plaque radius of 2 mm. For a plaque of thickness 1.5 mm and a phage concentration in the plaque of $10^{10}$/ml (30), $10^8$ phage were produced. Thus, the frequency of deletion is $10^{-8}$, consistent with values obtained in liquid culture (22).

## DISCUSSION

I traced the emergence of genetically distinct phage T7 mutants along different radii of a growing plaque. Unlike mutants of influenza A virus, which have been isolated from plaques without regard to spatial orientation (19), these phage mutants were significantly fitter than the wild type. It is not known, however, why their different rates of emergence evident in Fig. 2A and B were not reflected in the one-step growth curves of Fig. 3. Differences in the adsorption rate or affinity of the phage to its host may play a role. For example, fast or high-affinity adsorption, which is advantageous in liquid culture, may be advantageous or disadvantageous for propagation in a plaque, depending on the concentration of the host (31).

Although the conditions required for the generation of mutants await systematic study, a rule of thumb can be distilled from my experience. Higher host concentrations, attained by preparing plates with concentrated hosts and elevated nutrient concentrations, encourage the emergence of mutants. It is not known whether these conditions are inherently mutagenic or whether they influence the statistics by generating more phage.

The plaque evolution system provides an opportunity to test theoretical predictions on the behavior of evolving virus populations. For example, the quasi-species theory developed by Eigen and coworkers (9–11) predicts that the emergence and selection of favorable mutant populations should become more deterministic as population numbers are increased; one would then expect the distribution of mutant appearance times and selection profile shapes to narrow with increasing phage density. Further, by growing the phage in the presence of mutagens, one could decrease the fidelity of viral replication to the vicinity of the error threshold, the point at which information is effectively lost, and observe whether more robust mutant distributions arise, as theory predicts.

This work may also be readily extended in practical directions to test antiphage strategies. It will be interesting, for example, to see how phage in a growing plaque adapt in the presence of antibodies elicited against the phage, inhibitors of the host receptors, or host-expressed ribozymes or repressors targeted at components of the phage metabolism.

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